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Microbiomes of Unreactive and Pathologically Altered Ileocecal Lymph Nodes of Slaughter Pigs

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Microbe-laden dendritic cells are shifted to ileocecal lymph nodes (ICLNs), where microbes are concentrated and an adequate immune response is triggered. Hence, ICLNs are at a crucial position in immune anatomy and control processes of the local immune system. Pathological alterations in ICLNs, such as reactive hyperplasia, *lymphadenitis purulenta*, or *granulomatosa*, can harbor a multitude of pathogens and commensals, posing a potential zoonotic risk in animal production. The aim of this study was to characterize the microbial diversity of unreactive ICLNs of slaughter pigs and to investigate community shifts in reactive ICLNs altered by enlargement, purulence, or granulomatous formations. Pyrosequencing of 16S rRNA gene amplicons from 32 ICLNs yielded 175,313 sequences, clustering into 650 operational taxonomic units (OTUs). OTUs were assigned to 239 genera and 11 phyla. Besides a highly diverse bacterial community in ICLNs, we observed significant shifts in pathologically altered ICLNs. The relative abundances of *Cloacibacterium*- and *Novosphingobium*-associated OTUs and the genus *Faecalibacterium* were significantly higher in unreactive ICLNs than in pathologically altered ICLNs. Enlarged ICLNs harbored significantly more *Lactobacillus*- and *Clostridium*-associated sequences. Relative abundances of *Mycoplasma*, *Bacteroides*, *Veillonella*, and *Variovorax* OTUs were significantly increased in granulomatous ICLNs, whereas abundances of *Pseudomonas*, *Escherichia*, and *Acinetobacter* OTUs were significantly increased in purulent ICLNs ($P < 0.05$). Correlation-based networks revealed interactions among OTUs in all ICLN groups, and discriminant analyses depicted discrimination in response to pathological alterations. This study is the first community-based survey in ICLNs of livestock animals and will provide a basis to broaden the knowledge of microbe-host interactions in pigs.

Enteric microbiota of pigs strongly influence the maintenance of gastrointestinal and systemic health. The definition of the healthy enteric microbiota in pigs is crucial for the characterization of community perturbations, which can cause asymptomatic, clinical, or zoonotic disease in animals and, after transmission, in humans (1). A transmission of potential pathogens to humans can be caused by contamination events in food production occurring during slaughter but also during processing before pork is prepared by the consumer (2). The occurrence of microbes that are not part of routine clinical procedures in diagnostics in pigs is problematic, because the causative agent can escape detection if clinical signs of illness are rare or similar to symptoms deriving from a well-studied pathogenic agent (3). Hence, asymptomatic reservoirs of food-borne pathogens can lead to a serious risk to public health (4). Furthermore, pathogenic and potentially pathogenic microbes (e.g., *Campylobacter*, *Yersinia*, *Listeria*, and *Salmonella*) are known to have their primary ecological niche in the gastrointestinal tract (GIT) of clinically healthy animals (2, 5). Generally, risk hazards for meat production are hard to estimate, because contamination events in food industry can occur at each process step (6). Therefore, the definition of enteric microbial communities and the understanding of shifts associated with pathological alterations in the gut and in gut-associated adnexa, like mesenteric lymph nodes (MLNs), is essential for risk assessment. Ileocecal lymph nodes (ICLNs) in particular, which belong to MLNs and are located near the *plica ileocaecalis* in the abdominal cavity, are of the utmost importance for bacteriological analyses and diagnosis of pathogens in slaughter pigs (7).

The maintenance of gut health in livestock animals depends on the barrier function of the intestinal tract and local immune defense. The translocation process through the epithelium and the transport of pathogens and commensal microbes to MLNs is manifold, and different mechanisms have been described recently (8, 9). The dominant tolerogenic pathway is that antigens are captured in nonlymphatic tissues by dendritic cells (DC) (10). Via the afferent lymphatic vessels, DCs are transported to T-cell areas of draining MLNs, initiating an adequate immune response (11). MLNs concentrate commensals and pathogens and are at a pivotal position in immune anatomy and control processes, building an interface between the innate and adaptive parts of the local immune system (8, 10, 12). The commensal-laden dendritic cells usually do not penetrate further into the systemic circulation than into MLNs. This leads to protection by the systemic immune system against undesirable living microbiota (10). MLNs are also known to be habitats for bacteria (13–15). The first immune response of MLNs to harmful microbiota is a reactive hyperplasia characterized by enlargement, soft consistency, and a protruding surface in dissection (16). Pathogens such as *Salmonella*, *Mycobac-*

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terium, and *Lawsonia* are known to cause unspecific enlargement of MLNs (17–19). *Lymphadenitis purulenta* is described to be caused mainly by pyogenic microorganisms, e.g., *Staphylococcus*, *Streptococcus*, *Actinomyces*, or *Fusobacterium* (16, 20). *Lymphadenitis granulomatosa*, where granulomatous formations can be observed, is known to be caused by *Mycobacterium* or *Rhodococcus* (21, 22).

Until now, alteration-based shifts at the community level in MLNs have not been examined, and information about the microbial diversity in healthy MLNs is rare. Several culture-dependent bacterial isolates of lymph nodes were identified to belong to, for instance, *Bacillaceae*, *Enterobacteriaceae*, *Clostridiales*, *Corynebacterineae*, and *Mycobacteriaceae* (23, 24). To our knowledge, only one study is available that examines the microbial community in lymph nodes with high-throughput sequencing technologies (25). This study examined retropharyngeal lymph nodes from healthy mule deer and found a diverse microbiota, including representatives of all bacterial phyla.

Generally, the advent of high-throughput sequencing methods enables cost-efficient identification of entire microbial communities (including DNA fragments from dead and living organisms) in given habitats and has already led to important insights into porcine gastrointestinal microbiota (26–28). Here, we present the first study examining the diversity of bacteria in ICLNs of livestock animals and give deep insights into community shifts in ICLNs from slaughter pigs.

We hypothesized that ICLNs harbor a high bacterial diversity similar to that of the gastrointestinal community and that we would discover significant community shifts in pathologically altered lymph nodes, depending on their pathology. The major objectives of this study were to characterize the microbiome of unreactive ICLNs of slaughter pigs and to investigate community shifts in reactive lymph nodes showing enlargement, purulence, or granulomatous formations. Thirty-two unreactive and pathologically altered lymph nodes were taken from slaughter pigs, and the hypervariable V1-V2 region of the 16S rRNA gene was amplified. We obtained 175,313 16S rRNA gene sequences, representing an order of several magnitudes higher than findings of previous studies detecting microbes in lymph nodes. The diversity of microbes among ICLNs was demonstrated on operational taxonomic unit (OTU), genus, and phylum levels. In this study, we report a high diversity of bacteria in ICLNs of slaughter pigs. Depending on the pathological alterations of ICLNs, specific OTU abundance-based shifts could be observed. We could also describe significantly increased OTUs in pathologically altered ICLNs that have not been associated with being a causative agent or in cooccurrence with a causative agent of a pathological alteration before. Overall, this community description provides a basis for ecological understanding and bacterial translocation and may be used for risk assessment in pig slaughterhouses.

MATERIALS AND METHODS

Sampling and pathological alterations. ICLNs from pigs ($n > 300$) were taken at an IFS (International Food Standard)-certified slaughter house in Austria. After removing the gastrointestinal tract from the abdominal cavity of slaughter pigs, the *plica ileocaecalis* was disrupted and the local ICLN package was exposed by blunt preparation. The lymph nodes were removed from fat, vasculature, and connective tissue. All ICLNs deriving from one pig were packed in one plastic tube, cooled on ice, and transferred to the University of Veterinary Medicine, Vienna, Austria. Subsequently, ICLNs were purified from loose tissue and fat residues, and the surface was disinfected by rigorously dipping in 80% ethanol and flaming. Each ICLN was cut medially using sterile scalpel blades for visual inspection. Five groups were set up for the description of visual inspection: (i) unreactive ICLNs (healthy; no pathological alterations), (ii) enlarged ICLNs (more than 2-fold larger than the mean size of unreactive lymph nodes), (iii) granulomatous ICLNs (the lymphatic tissue includes granulomata), (iv) purulent ICLNs (the lymphatic tissue includes purulence or purulent spots), and (v) ICLNs with other pathological alterations (e.g., cystic formations or different alterations in one lymph node). Harvesting, preparation, and inspection of ICLNs was done by the same person. Eight lymph nodes were randomly chosen from the groups termed unreactive (healthy), enlarged, granulomatous, and purulent, considering the sample harvesting date and pig farmers' origins. In every group, ICLNs from at least two different sampling dates and of at least three different pig farmers had to be present, otherwise random selection was repeated. In total, 32 ICLNs were used for this study (8 ICLNs per group). Unreactive and pathologically altered ICLNs of slaughter pigs are depicted in Fig. 1.

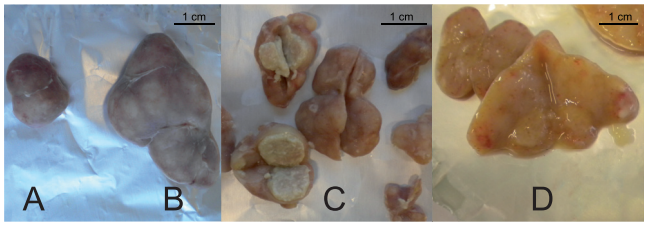


FIG 1 Unreactive and pathologically altered ICLNs of pigs. Unreactive ICLNs (A), enlarged ICLNs (more than 2-fold size compared to the mean size of unreactive lymph nodes) (B), granulomatous ICLNs (the lymphatic tissue includes granulomata) (C), and purulent ICLNs (the lymphatic tissue includes purulence or purulent spots) (D) are depicted. For collection of ICLNs, the *plica ileocaecalis* was disrupted, and the local lymph node package was bluntly prepared. Fat, vasculature, and connective tissues were removed.

DNA extraction and preparation of 16S rRNA gene amplicon libraries. Genomic DNA was extracted from 250 mg of ICLN tissue using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. The DNA concentration was determined using a Qubit fluorometer (Invitrogen, Carlsbad, CA). DNA was adjusted to 25 ng/μl in diethyl pyrocarbonate (DEPC)-treated water (Fermentas GmbH, St. Leon-Rot, Germany). 16S rRNA genes were amplified by using universal primers F27 (AGA GTT TGA TCC TGG CTC AG) (29) and R357 (CTG CTG CCT YCC GTA) (30) targeting the V1-V2 variable regions of the 16S rRNA gene (Lib-L kit, primer A-primer B; Roche 454 Life Science, Branford, CT). The 50-μl volume of the PCR mixture contained 1× fast start buffer; 2.5 U high-fidelity enzyme; 200 μM (each) dATP, dTTP, dGTP, and dCTP; 0.4 μM barcoded primers (Eurofins MWG, Ebersberg, Germany); 2.5 mM $MgCl_2$; and PCR-grade water (Roche Diagnostics, Mannheim, Germany). Barcoded primer sequences are listed in Table S5 in the supplemental material. Total genomic DNA (125 ng) was added to the PCR. Amplification in a standard thermocycler after initial denaturation at 95°C for 3 min was performed in 38 cycles at 95°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min with a final extension for 7 min. Amplicons were purified (Transgenomic Inc., Omaha, NE) and eluted by a linear gradient of acetonitrile in 0.1 M trimethyl ammonium acetate; amplicon DNA was purified subsequently on NucleoFast 96 PCR plates (Macherey-Nagel, Düren, Germany). The DNA concentrations were determined using a PicoGreen double-stranded DNA (dsDNA) assay kit (Life Technologies, Carlsbad, CA). Thirty barcode-labeled amplicons were pooled and analyzed using a 2100 Bio Analyzer (Agilent Technologies, Waldbronn, Germany). Emulsion PCR of pooled samples was performed with the GS titanium MV emPCR kit (Roche 454 Life Science).

Pyrosequencing, sequence processing, assignment, and statistics. Sequencing was performed with the GS-FLX titanium sequencing kit

XLR70 (Roche 454 Life Science) according to the manufacturer's instructions. Library preparation and sequencing were done at the Medical University of Graz (Center for Medical Research, Core Facility Molecular Biology, Austria). All reads derived from GS-FLX sequencing (32 samples; 175,313 reads) were processed with the software mothur, version 1.30.2 (31), according to the procedure from Schloss and Westcott (32). Low-quality sequences, primers, and barcodes were trimmed with the following parameters. The minimum average quality score was 35 (with a window size of 50 bp). The minimum length of reads was 162 bp, with an allowed number of differences from the primer sequence of 2. The maximal homopolymer length was eight, and the maximum number of differences from the barcode sequences was one. With the commands "pre.cluster" and "chimera.uchime," sequencing errors and chimeric sequences were excluded. A total of 121,700 sequences (69.4%) passed the quality control. For the classification of the remaining sequences, the RDP naïve Bayesian rRNA classifier (33) was used with the SILVA small-subunit reference database, v102 (34). Uncorrected pairwise distances were calculated with the command "dist.seqs" and were used as the input for the assignment to OTUs (operational taxonomic units). OTUs were assigned using a distance limit of 0.03. OTUs that contained fewer than five sequences assigned were removed. The "classify.otu" command is used to establish a consensus taxonomy for every OTU.

For estimation of total species diversity in lymph nodes, data were normalized. A random selection of the same number of sequences per sample was done; for this, the sequence number was based upon the sample with the lowest number of reads ($n = 1{,}095$ sequences). Species richness estimators, diversity indices, and Bray-Curtis similarity were calculated with "summary.single."

For generating summarized, smooth rarefaction curves per lymph node group, an algorithm previously described (35) was used. The assigned OTUs, classification to genus and phylum levels, and the diversity indices were subjected to analysis of variance (ANOVA) using PROC MIXED of SAS (Statistical Analysis System 9.2, SAS Institute Inc., Cary, NC). The SAS model included the option "fixed effects of lymph node group and sampling date," and the model considers ICLN an experimental unit. The covariance structure was modeled separately. Orthogonal contrasts were used to test a linear effect of the pathology and the effect of unreactive versus enlarged, granulomatous, and purulent ICLNs; unreactive and enlarged versus granulomatous and purulent ICLNs; enlarged versus unreactive, granulomatous, and purulent ICLNs; granulomatous versus unreactive, enlarged, and purulent ICLNs; or purulent versus unreactive, enlarged, and granulomatous ICLNs on response parameters. Means were reported as least-square means ± standard errors of the means (SEM). The procedure PROC MEANS of SAS was used for determination of lower and upper bounds of confidence intervals from diversity indices. The significance level was set to $P < 0.05$; $0.05 < P \leq 0.10$ was defined as a trend.

OTUs containing >250 sequences were blasted against NCBI GenBank nr (uncultured/environmental sample sequences were excluded in blast options). Fifty OTUs were blasted and linked with their closest reference strains, including accession numbers and sequence similarity. For the illustration of community shifts, discriminant analyses and pairwise correlation analyses were calculated in JMP Pro (SAS Institute, Cary, NC). Correlation networks were done with MENAP (molecular ecological network analysis pipeline [http://ieg2.ou.edu/MENA/]) (36). Networks were visualized with Cytoscape version 3.0.1 (37). Heat maps were created using JColorGrid (38).

Pyrosequence accession number. The pyrosequencing data are available in the EMBL SRA database under accession number ERP003687.

RESULTS

Tag pyrosequencing reveals a highly diverse microbial community in ICLNs of pigs. All reads derived from deep pyrosequencing of 32 ICLN samples were processed together. A total of 1,095 to 9,715 pyrotags per ICLN sample were generated. In total, 121,700 sequences (69.4%; mean length, 175 bp) passed the quality control. Considering all 32 samples, 650 OTUs were assigned containing more than 5 sequences per OTU (975 OTUs with fewer than 5 reads were excluded). This OTU classification was the basis for all further downstream analyses. Fifty OTUs contained more than 250 sequences (>0.2% relative abundance) and up to 23,021 sequences. These 50 OTUs are depicted in Table 1, including the closest reference strain and GenBank accession numbers. The closest reference strains of the OTUs were previously described to belong to the commensal GIT microbiota, plants, or environmental samples. Based on pathological alterations, ICLNs were sorted into four ICLN groups (unreactive, enlarged, granulomatous, and purulent) (Fig. 1). Species richness and diversity indices for ICLN groups are listed in Table S1 in the supplemental material. The abundance-based coverage estimator (ACE) significantly differed between enlarged and purulent ICLNs ($P < 0.01$ by PROC MIXED of SAS). The Simpson index (39) and the Shannon index (40) differed between granulomatous and purulent ICLNs (both indicated as a trend by PROC MIXED of SAS). In abundance curves (Fig. 2A), it is depicted that ICLN groups contained a low proportion of highly abundant OTUs (>5%) and a medium proportion of OTUs with relative abundances between 5 and 0.1%, whereas the bulk of the diversity consisted of very rare organisms (<0.1%). Twenty-two OTUs of unreactive ICLNs, 34 OTUs of enlarged ICLNs, 31 OTUs of granulomatous ICLNs, and 13 OTUs of purulent ICLNs reached relative abundances of >0.5%. Rarefaction curves (Fig. 2B) were calculated for all samples and are summarized as ICLN group means. High-diversity coverage was achieved, particularly for the purulent and granulomatous ICLN groups, with rarefaction curves reaching asymptotes. The tendency of purulent ICLNs toward decreased species richness was confirmed by ACE estimators. The decreased species richness of purulent ICLNs in Chao 1 estimations was not significant compared to levels for other ICLN groups. In Fig. 2C, the actual number of OTUs in ICLNs after quality control is shown. The median values for OTUs observed in unreactive, enlarged, purulent, and granulomatous ICLNs were 83.5, 92, 35.5, and 79.5, respectively. The lower diversity of purulent ICLNs was not statistically significant ($P < 0.01$ by PROC CORR; data not shown).

Microbial communities exhibit distinct abundance patterns according to pathological alterations of ICLNs. The Venn diagram of Fig. 2D displays a high number of unique OTUs per ICLN group but also shows clear overlap patterns for all groups. From 650 OTUs assigned throughout all samples, 59 OTUs (9%) were shared between all ICLN groups. The highest degree of similarity between ICLN groups was found for unreactive versus enlarged and unreactive versus granulomatous ICLNs, where 169 and 139 OTUs (63 and 52% of total OTUs from unreactive ICLNs), respectively, could also be detected in enlarged or granulomatous ICLN. Purulent ICLNs had low similarity to other ICLN groups (38% similarity to unreactive ICLNs, 22% similarity to enlarged and to granulomatous ICLNs). This was confirmed by Bray-Curtis similarity, showing clear clustering of purulent ICLNs versus other ICLN groups and indicating distinct microbial community structures of purulent ICLNs. The other ICLN groups also included 2 to 3 ICLNs with high similarity, showing overlapping community patterns between some ICLNs of different ICLN groups (see Fig. S1 in the supplemental material). In the discriminant analyses using the first 3 principal components, it could be

TABLE 1 Fifty most abundant OTUs for all ICLN groups

OTU	No. of reads	Relative abundance (%)	Closest reference strain[a]	Similarity (%)
2	23,021	19.2	*Pseudomonas lurida* (HE716902)	100
15	13,717	11.4	*Escherichia coli* (JN713442)	100
4	8,094	6.7	*Acidovorax ebreus* (NR074591)	100
240	7,795	6.5	*Carnobacterium divergens* (FJ656706)	99
5	4,478	3.7	*Terrahaemophilus aromaticivorans* (GU428288)	100
3	3,383	2.8	*Clostridium lituseburense* (M59107)	97
8	2,637	2.2	*Bacteroides dorei* (AB242143)	100
6	2,614	2.2	*Propionibacterium acnes* (JF277163)	100
217	2,346	2.0	*Mycoplasma hyosynoviae* (NR029183)	99
168	1,976	1.6	*Brochothrix thermosphacta* (AB680248)	99
32	1,827	1.5	*Faecalibacterium prausnitzii* (HQ457032)	97
13	1,701	1.4	*Cloacibacterium rupense* (HF937051)	100
17	1,667	1.4	*Faecalibacterium prausnitzii* (HQ457032)	99
60	1,633	1.4	*Bacteroides fragilis* (AB618792)	99
7	1,520	1.3	*Clostridium cocleatum* (NR026495)	89
1	1,469	1.2	*Turicibacter sanguinis* (HQ428099)	99
169	1,310	1.1	*Psychrobacter fozii* (KF054907)	99
33	1,100	0.9	*Clostridium bartlettii* (NR027573)	99
11	1,087	0.9	*Clostridium paraputrificum* (X73445)	99
47	1,029	0.9	*Stenotrophomonas rhizophila* (HG421016)	100
274	866	0.7	*Fusobacterium nucleatum* (JN713538)	100
44	788	0.7	*Veillonella parvula* (GU406730)	100
14	759	0.6	*Novosphingobium panipatense* (NR044210)	99
268	744	0.6	*Pseudomonas putida* (KC195888)	100
181	693	0.6	*Staphylococcus haemolyticus* (KC329826)	99
75	632	0.5	*Serratia proteamaculans* (JN859195)	100
10	581	0.5	*Lactobacillus amylovorus* (EF120375)	99
587	572	0.5	*Acinetobacter junii* (KF055000)	99
54	568	0.5	*Pseudomonas aeruginosa* (KC570343)	99
275	560	0.5	*Leptotrichia trevisanii* (AY029801)	99
218	554	0.5	*Mycobacterium avium* subsp. *hominissuis* (AP012555)	99
9	547	0.5	*Clostridium disporicum* (NR026491)	99
222	497	0.4	*Leucobacter komagatae* (KC845231)	99
18	490	0.4	*Klebsiella pneumoniae* (KF192506)	99
26	489	0.4	*Klebsiella oxytoca* (HF678365)	99
23	465	0.4	*Bacteroides dorei* (AB714352)	99
170	461	0.4	*Psychrobacter cibarius* (AY639872)	99
276	399	0.3	*Campylobacter gracilis* (JX912515)	99
25	398	0.3	*Clostridium glycolicum* (EU887819)	99
180	390	0.3	*Variovorax paradoxus* (AB627010)	99
1161	390	0.3	*Fusobacterium necrophorum* (JN713357)	99
19	366	0.3	*Bacteroides uniformis* (AB510711)	99
34	359	0.3	*Comamonas denitrificans* (DQ836252)	98
690	323	0.3	*Carnobacterium divergens* (FJ656710)	99
175	321	0.3	*Rhodococcus qingshengii* (KF055006)	100
73	297	0.2	*Ruminococcus gnavus* (EU139255)	100
237	275	0.2	*Comamonas testosteroni* (AB680220)	99
12	260	0.2	*Clostridium disporicum* (NR_026491)	98
183	256	0.2	*Blautia wexlerae* (NR044054)	99
118	253	0.2	*Serratia marcescens* (KF054974)	100

[a] GenBank accession numbers are shown in parentheses. BLASTN against GenBank nr excluded uncultured sample sequences.

shown that all ICLN groups are discriminated from each other in response to pathological alterations (see Fig. S2).

In Fig. 3A, relative abundances of the 50 most abundant OTUs are shown. ICLN groups had diverse abundance shifts throughout the 50 most abundant OTUs. Interestingly, every ICLN group showed characteristic peaks, suggesting a unique abundance pattern for every ICLN group. In Fig. 3B, the distribution and representation of the best blast hits (NCBI) of the 50 most abundant OTUs are visualized (exact abundance values and SEM are listed in Table S2 in the supplemental material). All ICLN groups harbored 2 OTUs with a relative abundance of more than 5%: OTU 2 (best blast hit, *Pseudomonas lurida*) and OTU 15 (best blast hit, *Escherichia coli*). Additionally, several OTUs were highly abundant (>5%) in only one or two ICLN groups. In unreactive and enlarged ICLNs, OTU 4 (best blast hit, *Acidovorax ebreus*), was highly abundant, whereas OTU 5 (best blast hit, *Terrahaemophilus aromaticivorans*) was highly abundant only in unreactive ICLNs. OTU 240 in enlarged and purulent ICLNs (best blast hit, *Carno-*

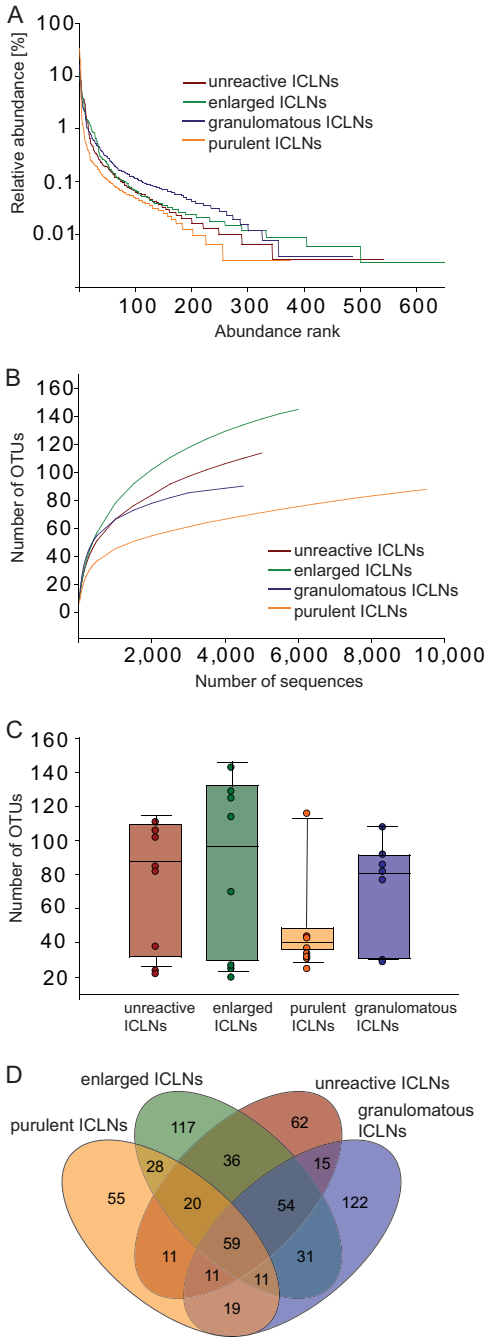
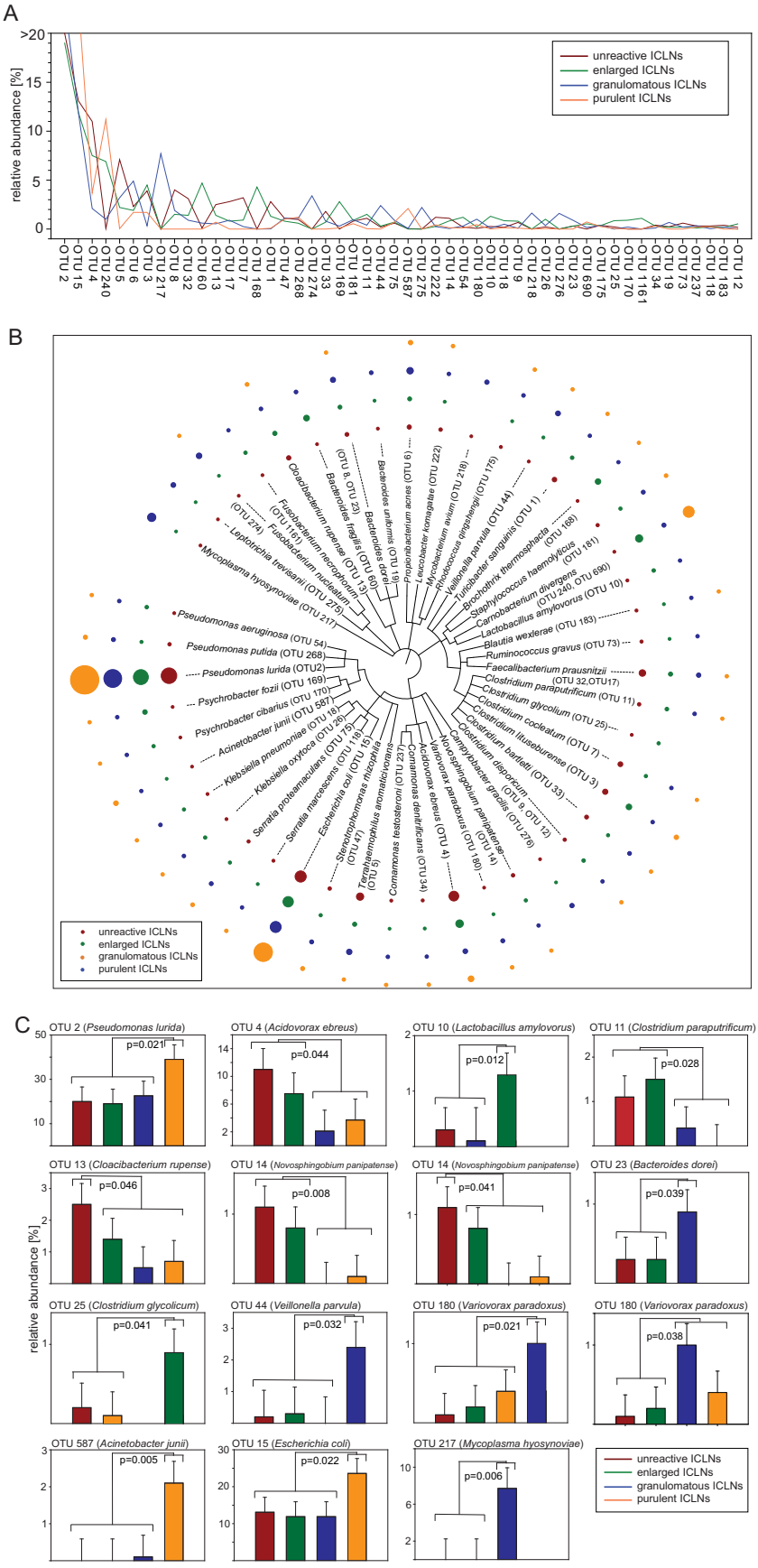


FIG 2 Diversity of microbial communities in ileocecal lymph nodes of pigs. Rank abundance (A) and rarefaction curves (B) were based on an OTU distance limit of 0.03. Read numbers of samples were normalized (random selection) prior to calculation. Curves were calculated for each sample and are depicted as means per lymph node group (unreactive, enlarged, granulomatous, and purulent ICLNs). (C) Numbers of OTUs detected in ICLNs are given per lymph node group. (D) The Venn diagram depicts the number of shared OTUs between lymph node groups.

bacterium divergens) and OTU 217 in granulomatous ICLNs (best blast hit, *Mycoplasma hyosynoviae*) were highly abundant. In Fig. 3C, all significant shifts of OTUs according to ICLN groups are shown. OTU 13 and OTU 14 (best blast hits, *Cloacibacterium rupense* and *Novosphingobium panipatense*, respectively) were significantly increased in unreactive ICLNs compared to other ICLN groups (2.7- and 3.7-fold changes, respectively). OTU 4, OTU 11, and OTU 14 (best blast hits, *Acidovorax ebreus*, *Clostridium paraputrificum*, and *Novosphingobium panipatense*, respectively) were significantly increased, whereas OTU 180 (best blast hit, *Variovorax paradoxus*) was significantly decreased in unreactive and enlarged ICLNs compared to granulomatous and purulent ICLNs (3.2-, 6.5-, 19-, and 4.7-fold changes, respectively). In enlarged ICLNs, OTU 10 and OTU 25 (best blast hits, *Lactobacillus amylovorus* and *Clostridium glycolicum*, respectively) were significantly increased compared to other ICLN groups (3.3- and 3-fold change). OTU 23, OTU 44, OTU 180, and OTU 217 (best blast hits, *Bacteroides dorei*, *Veillonella parvula*, *Variovorax paradoxus*, and *Mycoplasma hyosynoviae*, respectively) were significantly increased in granulomatous ICLNs compared to other ICLN groups (4.5-, 15-, 4-, and 7.7-fold change, respectively). OTU 2, OTU 15, and OTU 587 (best blast hits, *Pseudomonas lurida*, *Escherichia coli*, and *Actinetobacter junii*, respectively) were significantly increased in purulent ICLNs compared to other ICLN groups (1.9-, 1.6-, and 21-fold change, respectively).

Correlation networks and pairwise correlations reveal changes of interactions in ICLN groups. To reveal interactions among OTUs, networks based on the correlation between OTUs were built for all ICLN groups ($r > 0.7$; $P < 0.02$) (see Fig. S3 in the supplemental material). In addition, pairwise correlations were calculated for the 20 most abundant OTUs per ICLN group, respectively. In the network topology, correlations were found in all ICLN groups, with a remarkably high correlation pattern in unreactive and enlarged ICLNs that declined in frequency in granulomatous and especially in purulent ICLNs (see Fig. S4). Pairwise correlation analyses revealed very high correlations ($r > 0.9$) between OTU 4 (best blast hit, *Acidovorax ebreus*) and OTU 13 (best blast hit, *Cloacibacterium rupense*) in all ICLN groups, indicating a very strong pattern of cooccurrence. In unreactive and purulent ICLNs, OTU 2 (best blast hit, *Pseudomonas lurida*) and OTU 15 (best blast hit, *Escherichia coli*) correlated highly ($r > 0.9$), whereas in unreactive, enlarged, and granulomatous ICLNs, OTU 7 (best blast hit, *Clostridium* sp.) and OTU 32 (best blast hit, *Faecalibacterium prausnitzii*) were highly correlated ($r > 0.9$). In unreactive, enlarged, and purulent ICLNs, OTU 1 (best blast hit, *Turicibacter sanguinis*) and OTU 3 (best blast hit, *Clostridium lituseburense*) were highly correlated ($r > 0.9$).

Distinct OTU patterns dependent on pathological alterations of ICLNs are identified to genus level. In pairwise correlation analyses (see Fig. S4 in the supplemental material), it was observed that highly abundant OTUs belonging to the same genus did not correlate in the same ICLN group. Hence, these OTUs were interchangeable within high-abundance genera. Therefore, analyses on the genus level seem to be ecologically worthwhile, deepening the understanding of bacterial translocation and DC sampling. Nevertheless, it should be kept in mind that different OTUs within a genus might have different functional and zoonotical potential. For analyses, we assigned all OTUs to their respective genera to get insights into accumulated shifts dependent on the pathological alteration. In total, 650 OTUs were assigned to 239 genera. In Fig. 4A the core microbiome of unreactive ICLNs, including genera with >1% relative abundance (22 genera) and phylum affiliation, is visualized. The proteobacteria *Pseudomonas*, *Escherichia-Shigella*, *Acidovorax*, and *Haemophilus* were the most abundant genera (24, 13.2, 11, and 7.1% of all sequences, respec-


A
relative abundance [%]
>20
15
10
5
0
unreactive ICLNs
enlarged ICLNs
granulomatous ICLNs
purulent ICLNs
OTU 2
OTU 15
OTU 4
OTU 240
OTU 5
OTU 6
OTU 3
OTU 217
OTU 8
OTU 32
OTU 60
OTU 13
OTU 17
OTU 7
OTU 168
OTU 1
OTU 47
OTU 268
OTU 274
OTU 33
OTU 169
OTU 181
OTU 11
OTU 44
OTU 75
OTU 587
OTU 275
OTU 222
OTU 14
OTU 54
OTU 180
OTU 10
OTU 18
OTU 9
OTU 218
OTU 26
OTU 276
OTU 23
OTU 690
OTU 175
OTU 25
OTU 170
OTU 1161
OTU 34
OTU 19
OTU 73
OTU 237
OTU 118
OTU 183
OTU 12
B
Mycoplasma hyosynoviae (OTU 217)
Leptorichia trevisanii (OTU 275)
Fusobacterium nucleatum (OTU 274)
Fusobacterium necrophorum (OTU 161)
Cloacibacterium rupense (OTU 13)
Bacteroides fragilis (OTU 60)
(OTU 8, OTU 23)
Bacteroides dorei
Bacteroides uniformis (OTU 19)
Propionibacterium acnes (OTU 6)
Leucobacter komagatae (OTU 222)
Rhodococcus aichiensis (OTU 218)
Mycobacterium avium (OTU 175)
Rhodococcus gingshengii
Veillonella parvula (OTU 44)
Turicibacter sanguinis (OTU 1)
Brochothrix thermosphacta (OTU 168)
Staphylococcus haemolyticus (OTU 181)
Staphylococcus divergens (OTU 240, OTU 690)
Carnobacterium amylovorus (OTU 10)
Lactobacillus amylovorus (OTU 183)
Blautia wexlerae (OTU 73)
Ruminococcus gravus (OTU 73)
Faecalibacterium prausnitzii (OTU 32,OTU17)
Clostridium paraputrificum (OTU 11)
Clostridium glycolicum (OTU 25)
Clostridium cocleatum (OTU 7)
Clostridium lituseburense (OTU 3)
Clostridium bartlettii (OTU 33)
Clostridium disporicum (OTU 9, OTU 12)
Campylobacter gracilis (OTU 276)
Novosphingobium panipatense (OTU 14)
Variovorax paradoxus (OTU 180)
Acidovorax ebreus (OTU 4)
Comamonas denitrificans (OTU 34)
Comamonas testosteroni (OTU 217)
Terrahaemophilus aromaticivorans (OTU 5)
Sterolibacterium denitrificans (OTU 47)
Escherichia coli (OTU 15)
Serratia marcescens (OTU 18)
Serratia proteamaculans (OTU 75)
Klebsiella oxytoca (OTU 26)
Klebsiella pneumoniae (OTU 18)
Acinetobacter junii (OTU 587)
Psychrobacter cibarius (OTU 170)
Psychrobacter fozii (OTU 169)
Pseudomonas lurida (OTU2)
Pseudomonas putida (OTU 268)
Pseudomonas aeruginosa (OTU 54)
unreactive ICLNs
enlarged ICLNs
granulomatous ICLNs
purulent ICLNs
C
relative abundance [%]
OTU 2 (Pseudomonas lurida)
p=0.021
OTU 4 (Acidovorax ebreus)
p=0.044
OTU 10 (Lactobacillus amylovorus)
p=0.012
OTU 11 (Clostridium paraputrificum)
p=0.028
OTU 13 (Cloacibacterium rupense)
p=0.046
OTU 14 (Novosphingobium panipatense)
p=0.008
OTU 14 (Novosphingobium panipatense)
p=0.041
OTU 23 (Bacteroides dorei)
p=0.039
OTU 25 (Clostridium glycolicum)
p=0.041
OTU 44 (Veillonella parvula)
p=0.032
OTU 180 (Variovorax paradoxus)
p=0.021
OTU 180 (Variovorax paradoxus)
p=0.038
OTU 587 (Acinetobacter junii)
p=0.005
OTU 15 (Escherichia coli)
p=0.022
OTU 217 (Mycoplasma hyosynoviae)
p=0.006
unreactive ICLNs
enlarged ICLNs
granulomatous ICLNs
purulent ICLNs

tively). Interestingly, the most abundant genera consist of only one highly abundant OTU, except *Bacteroides* and *Clostridium* XI, which consist of several moderately abundant OTUs. In Fig. 4B, the ICLN relative abundances of the top 30 genera overall are depicted, and shifts dependent on pathological alterations were visualized. Exact values, SEM, and *P* values are available in Table S3. For 53% of the 30 most abundant genera, statistically significant shifts or trends in the relative abundances among ICLN groups were observed. *Faecalibacterium* and *Cloacibacterium* were significantly increased in unreactive ICLNs compared to all other ICLN groups (2.8-fold change). Enlarged ICLNs did not differ significantly from all other ICLN groups, but enlarged and unreactive ICLNs showed a significant increase of *Acidovorax* (3.2-fold change) compared to granulomatous and purulent ICLN groups. Granulomatous ICLNs harbored a significantly increased ratio of *Mycoplasma* (7.7-fold change) and *Veillonella* (9.6-fold change) compared to the other ICLN groups. The most significant changes could be described for purulent ICLNs versus other ICLN groups: *Pseudomonas*, *Escherichia-Shigella*, and *Acinetobacter* were significantly increased in purulent ICLNs (1.8-, 1.6-, and 5.4-fold change, respectively).

***Proteobacteria* dominate in all ICLN groups.** Throughout all OTUs of ICLNs, 11 phyla were identified, with *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* being the most abundant ones (84% of all reads). In all ICLN groups, *Proteobacteria* was the dominating phylum (>50% of all reads), followed by *Firmicutes* (15 to 33%) and *Bacteroidetes* (4 to 11%). Unreactive, enlarged, and purulent ICLN groups did not significantly differ in phylum abundances. In granulomatous ICLNs, *Synergistetes*, *Actinobacteria*, and *Tenericutes* significantly increased (15-, 2.5-, and 7.3-fold change, respectively) compared to other ICLN groups. Phylum abundances are depicted in Fig. S5 and are listed in detail in Table S4 in the supplemental material.

DISCUSSION

In this study, we examined the microbiome of ICLNs of slaughter pigs using culture-independent methods. This study provides the first deep-sequencing survey of the diversity in unreactive and pathologically altered ICLNs of slaughter pigs. It revealed characteristic changes in the composition of bacteria depending on pathological alterations in ICLNs. Due to the immense complexity of the porcine gut microbiota from which DCs are sampling microbes and subsequently shifting them to ICLNs, effects of abundance changes would not have been evident to the same extent by less powerful methods. In the present study, we describe three major findings. (i) A highly diverse bacterial community in ICLNs of slaughter pigs, which has not been reported before. (ii) ICLN groups differing in pathology had distinct OTU abundance-based patterns with significant shifts in abundances compared to other ICLN groups. (iii) Besides microorganisms that are known to be responsible for specific pathological alteration of the lymph node, we observed significantly increased OTUs in ICLN groups that were not described to be a causative agent or in cooccurrence with a causative agent of pathological alterations before. Matches of OTUs to the GenBank nr with high similarity approximate species-level taxonomy, but it should be kept in mind that the average read length of 175 bp is too short to reliably classify OTUs on the species level.

High diversity in the ICLN microbiome. The ICLN microbiome contained a diverse representation among microbes from different phyla, e.g., *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*, which are known to be highly abundant in the gastrointestinal tract of pigs (26, 28). In our study, 650 OTUs from 11 different phyla were detected, indicating a diversity similar to that of mucosal sites and luminal digesta of the porcine gastrointestinal tract, where between 127 and 997 OTUs could be assigned (28 and E. Mann, S. Schmitz-Esser, Q. Zebeli, M. Wagner, M. Ritzmann, and B. U. Metzler-Zebeli, unpublished data). Interestingly, ICLNs harbored a higher number of genera than was found in studies of different gastrointestinal sites in pigs (26, 28), which might be explained by the long-time storage of microbes and DNA fragments in lymph nodes. Because our methodology was based on DNA amplification, we could not distinguish between living bacteria and DNA fragments that have been shifted to ICLNs; this will be an interesting additional approach in the future. All closest reference strains of the 50 most abundant OTUs examined with a blast search against GenBank nr have been previously described to belong to the commensal GIT microbiota or environmental samples. Interestingly, the highly abundant OTU 4, assigned to *Acidovorax ebreus*, which was not associated with pig intestinal commensals until now, was recently described in lymph nodes of mule deer (25) and is specialized for survival in complex ecosystems (41). Generally, the diversity of the community detected in our study was comparable to that of healthy retropharyngeal lymph nodes of mule deer (25), although host species, and area of innervation, and location of the lymph nodes were different. Further studies are needed for detailed interspecial and translocal lymph node comparisons.

Unraveling distinct microbial patterns of ICLNs. Every ICLN group had distinct OTU and genus abundance-based patterns with significant shifts in OTU abundances.

(i) Unreactive ICLNs. The significant increase of the genus *Faecalibacterium* OTU 13 and OTU 14 (best blast hits, *Cloacibacterium rupense* and *Novosphingobium panipatense*) in unreactive versus other ICLN groups indicate that an increased abundance of these bacteria is associated with a balanced community in ICLNs. Until now, these bacteria were not described in ICLN of pigs, although these genera have been found frequently in mammal's guts and at mucosal surfaces (42, 43). *Faecalibacterium*, one of the most abundant butyrate producers in the gastrointestinal tract, was recently described to serve as an indicator of intestinal homeostasis (44). The significant increase of this genus in unreactive, healthy ICLNs is a first indication of an association between the

FIG 3 Relative abundances of OTUs in ileocecal lymph nodes of pigs and shifts dependent on the pathological alterations of ileocecal lymph nodes. (A) For unreactive, enlarged, granulomatous, and purulent ICLNs, the 50 most abundant OTUs are shown. (B) Distribution and representation of the best blast hits (GenBank nr) and relative abundance of the 50 most abundant OTUs (OTUs are listed in parentheses) dependent on pathological alterations are visualized. The size of colored circles indicates the relative abundance of OTUs in the data set. Phylogenetic relationships of best blast hits (GenBank nr) are denoted. The figure was created with iTOL (62). (C) Shifts induced by ICLN groups on the OTU level that reached statistical significance ($P < 0.05$) are shown. Best blast hits (GenBank nr) are shown in parentheses.

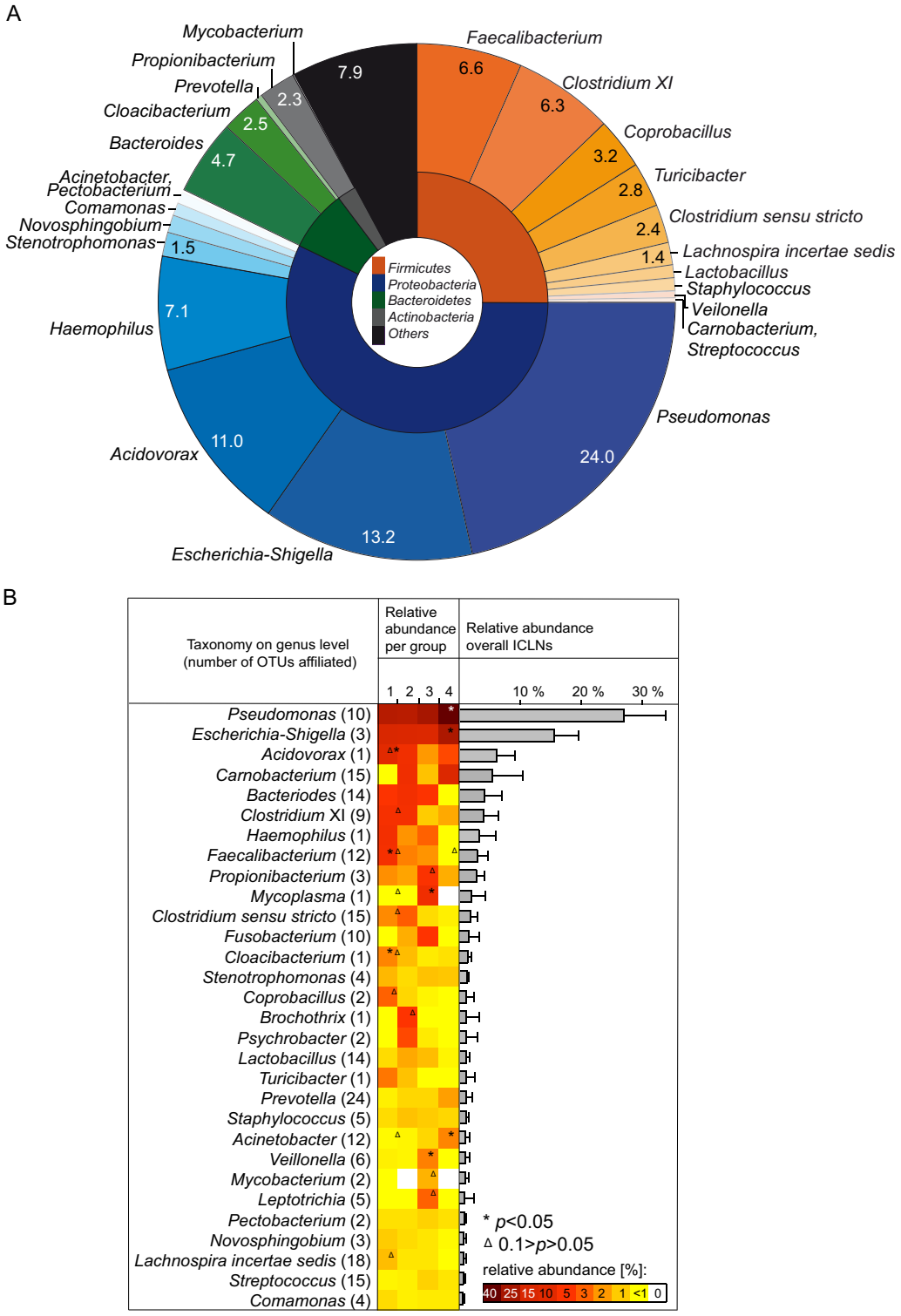


FIG 4 Relative abundances of genera in ileocecal lymph nodes of pigs. (A) Visualization of the core microbiome in unreactive ICLNs, including genera with >1% relative abundance and phylum affiliation. Relative abundances are given in percentages. (B) Relative abundances of the 30 most abundant genera are depicted for all ICLN groups, and SEM are included. ICLN groups: 1, unreactive; 2, enlarged; 3, granulomatous; and 4, purulent. Significant contrasts of ICLN groups and trends were marked in the heat map with an asterisk and triangle, respectively. Genus-level analyses were based on OTU classification. The number of OTUs affiliated with a genus is shown in parentheses.

high abundance of *Faecalibacterium* (6.6%) and a dynamic microbial equilibrium in ICLNs.

(ii) Enlarged ICLNs. Enlarged ICLNs are often a preliminary stage of serious pathological alterations, reacting to harmful microbiota with a first immune response. *Salmonella* or *Lawsonia* have been described to cause unspecific enlargement of MLNs (18, 19). In our study, OTU 10 and OTU 25 (best blast hits, *Lactobacillus amylovorus* and *Clostridium glycolicum*) were significantly increased in enlarged ICLNs. Keeping in mind that having a higher abundance compared to other ICLN groups is not proof for being the causative agent of a pathological alteration, *Clostridium* species have already been isolated from deep neck abscesses that also caused an inflammatory response in lymph nodes (45). *Lactobacillus amylovorus* is known to be predominant in the porcine GIT (46), being involved in starch degradation in the upper GIT (47), but it has not been related to inflammatory processes in lymph nodes before. Interestingly, correlation analyses between OTU 10 (best blast hit, *Lactobacillus amylovorus*) and OTU 25 (*Clostridium glycolicum*) revealed high correlation ($r = 0.84$) in enlarged ICLNs (data not shown), indicating a cooccurrence or cross-talk pattern of these OTUs.

(iii) Granulomatous ICLNs. In our study, OTU 23, OTU 44, OTU 180, and OTU 217 (best blast hits, *Bacteroides dorei*, *Veillonella parvula*, *Variovorax paradoxus*, and *Mycoplasma hyosynoviae*, respectively) were significantly increased in granulomatous ICLNs compared to levels in other ICLN groups (4.5-, 15-, 4-, and 7.7-fold change, respectively). On the genus level, *Mycoplasma* and *Veillonella* increase could be significantly identified. Granulomas in ICLNs were described to be caused by *Mycobacterium* and *Rhodococcus* (21, 22), but *Veillonella*, *Mycoplasma*, *Variovorax*, and *Bacteroides* have never been associated with these pathological alterations. In our study, we assumed that granulomatous alterations were caused by OTU 218 (best blast hit, *Mycobacterium hyosynoviae*), but it should be mentioned that this OTU could also be detected in other ICLN groups in very low abundance. The increase of *Mycobacterium* in granulomatous ICLNs was not statistically significant but indicated a trend ($P = 0.05$). Because of the significant increase of *Veillonella*-, *Variovorax*-, *Mycoplasma*-, and *Bacteroides*-associated OTUs, we hypothesize that these OTUs have an unknown nexus (e.g., cooccurrence, cross-feeding, or direct influence) with the causative agent or the pathology of granulomatous formations. *Bacteroides* species, the most predominant anaerobes in the gut, are known to cause pathological alterations in tissues, including abscess formation in multiple body sites (e.g., the abdomen) as well as bacteremia (48). *Variovorax*, a common plant symbiont in the rhizosphere with diverse metabolic capabilities, was not described to act as a pathogen in mammals. However, it also has been isolated from retropharyngeal lymph nodes of mule deer (25, 49). *Veillonella parvula* is known to be a commensal of the normal gut microbiota but also is associated with bacteremia (50). However, concerning the granulomatous alterations of ICLNs, further investigations and community assignments are needed to provide evidence for a relationship between these OTUs and the pathology of ICLNs.

(iv) Purulent ICLNs. In purulent ICLNs, levels of OTU 2, OTU 15, and OTU 587 (best blast hits, *Pseudomonas lurida*, *Escherichia coli*, and *Acinetobacter junii*, respectively) were significantly increased compared to those of other ICLN groups (1.9-, 1.6-, and 21-fold change, respectively). These shifts were statistically approved by analyses on the genus level. *Escherichia* species are highly abundant gut commensals in pigs, whereas *Pseudomonas* species occur in smaller numbers in the porcine gut (46, 51). Surprisingly, in the ICLNs, *Escherichia*- and *Pseudomonas*-associated OTUs were the most abundant OTUs, with >30% of all reads generated in this study. *Escherichia* and *Pseudomonas* are known to be pyogenic organisms, as is *Acinetobacter junii*, having already been isolated from purulent tissues and lymph fluid (52, 53). This is the first time *Escherichia*, *Pseudomonas*, and *Acinetobacter* species have been associated with purulence in porcine ICLNs. Until now, *Staphylococcus*, *Streptococcus*, *Actinomyces*, and *Fusobacterium* were listed as causative agents for pus formation in lymph nodes (16, 20). Correlation networks revealed strong correlations of several OTUs in all ICLN groups, with the denseness of the correlation network decreasing from unreactive via enlarged and granulomatous to purulent ICLNs. In particular, purulent ICLNs lacked substantial correlation patterns, confirming the observation on the OTU level that these ICLNs were out of balance and loaded with a few very highly abundant OTUs.

We applied a multiplicity of analyses that permitted characterization of microbial patterns related to pathological alterations of ICLNs. Generally, we could identify several potential pathogens in all ICLN groups, e.g., OTU 218 (best blast hit, *Mycobacterium avium*), OTU 54 (best blast hit, *Pseudomonas aeruginosa*), OTU 6 (best blast hit, *Propionibacterium acnes*), and OTU 217 (best blast hit, *Mycoplasma hyosynoviae*). *Mycobacterium avium* is a serious health risk for immunocompromised humans and is associated with Crohn's disease (54, 55). It should be kept in mind that *Mycobacteria* also could be detected in ICLN groups other than granulomatous ICLNs in very low abundance, indicating a risk even if no granulomatous pathology can be observed in ICLNs. A similar observation was recently described in a study where 31% of *Mycobacterium*-positive lymph nodes of slaughter pigs did not show any visible lesions (56). This is interesting, considering that *Mycobacteria* are usually diagnosed only during postmortem examination in the slaughter process (57, 58). The presence of *Mycobacteria* in groups other than granulomatous ICLNs might represent an early stage of pathological alteration which is not yet morphologically identifiable. *Pseudomonas* spp. were recently detected in fresh pork products (59). The high relative abundance (24% of all reads) of the genus *Pseudomonas* suggests ICLNs of pigs are a contamination source. *Propionibacterium acnes* accounts for half of the human skin commensals (60); thus, we do not assume a zoonotic risk emanating from ICLNs of slaughter pigs. *Mycoplasma hyosynoviae* is recognized as the causative agent of enzoonotic pneumonia inducing mild respiratory symptoms in pigs (61), but it is also described to cause the porcine respiratory disease complex (PRDC) together with secondary pathogens. This complex is a serious pig disease worldwide, causing significant economic losses.

Further studies using RNA-based approaches might give insights into the viability of pathogens in ICLNs and contribute to a valid risk assessment in pork production and processing. Also, in future studies, the relatedness between the bacterial community in ICLNs and the gut bacterial community should be characterized to link the pathological condition of ICLNs to overall gut health. In conclusion, the present data provide the first deep insights into the community composition of ICLNs in slaughter pigs, showing highly diverse and complex bacterial communities in ICLNs. In particular, shifts in OTUs provide a basis to improve our under-

standing of bacterial associations in unreactive and pathologically altered ICLNs in pigs.

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